OLIGONUCLEOTIDE MANUFACTURING SCALING CHALLENGES

for Undruggable Targets

By Claire MacLeod, PhD

Oligonucleotide therapies hold great promise for treating a wide range of diseases, including the undruggable, but their journey from lab to patient hinges on fundamental manufacturing challenges that the industry is racing to solve.

"Undruggable" targets are proteins that resist conventional small molecule drugs and play a role in disease. These undruggable targets are found across multiple therapeutic areas, from rare to prevalent diseases. Oligonucleotides are a new class of therapeutics that leverage our understanding of the human genome and the development of the chemical synthesis of DNA to treat these previously undruggable targets.

The combination of complex chemical synthesis with biologics-like downstream processing and product processing presents manufacturing challenges. The oligonucleotide synthesis process requires multiple sequential chemical reactions, all conducted in large volumes of solvent. The oligonucleotide API manufacturing facility must be able to support the large volume of solvents and associated flammability hazards. As the process moves into downstream purification, the design considerations change focus to being able to meet low bioburden and endotoxin limits while dealing with large volume requirements of high-quality water. The facility design, process development, and manufacturing teams all need to have the required knowledge to support these two distinct areas. This is reflected in the European Medicines Agency (EMA) guidance that calls for consideration from both small molecules and biologics manufacturing to be included in the control strategy [1].

With the increase in demand for both the number and scale of oligonucleotide manufacturing set to increase over the next years [2], improvements to manufacturing technologies originating from both small molecule and biologics fields, and beyond, will be required so that the undruggable targets can be drugged with medicines that are cost and resource-use effective.

INTRODUCTION TO OLIGONUCLEOTIDES

Oligonucleotides are short strands of DNA and/or RNA. They are short (bio)polymers of nucleotide building blocks with typically 16–30 nucleotide units in the chain. Each nucleotide subunit consists of one of the four nucleobases and a ribose sugar and are linked together by a phosphodiester backbone. The structure of an oligonucleotide is shown in Figure 1.

Figure 1: Chemical structure of two nucleotide subunits in an oligonucleotide.

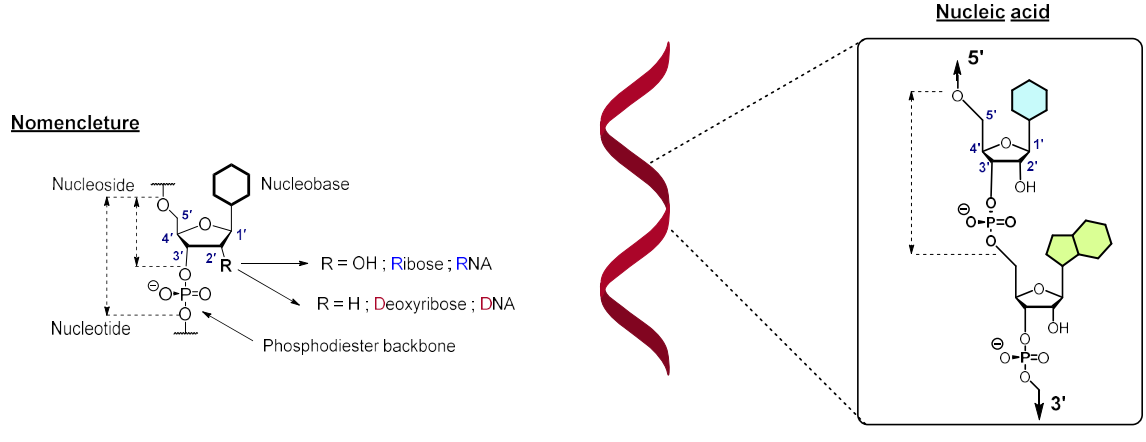


Figure 2: Block diagram of the oligonucleotide drug substance manufacturing process. Operations highlighted in orange are conducted in highly automated proprietary equipment; those in blue use standard pharmaceutical plant equipment (e.g., stirred mixed vessels).

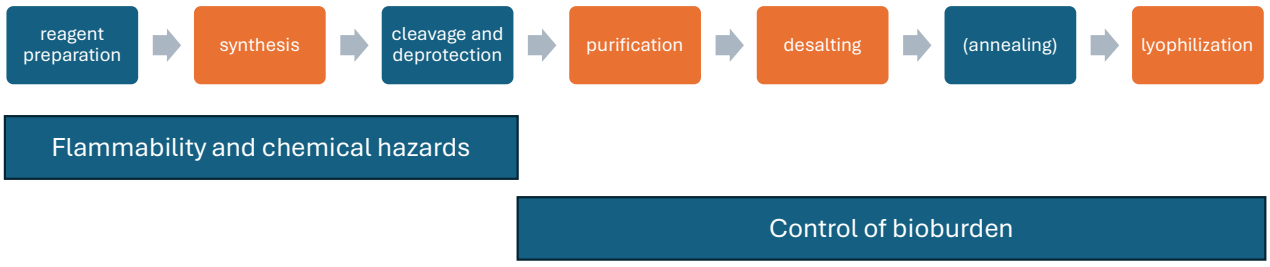
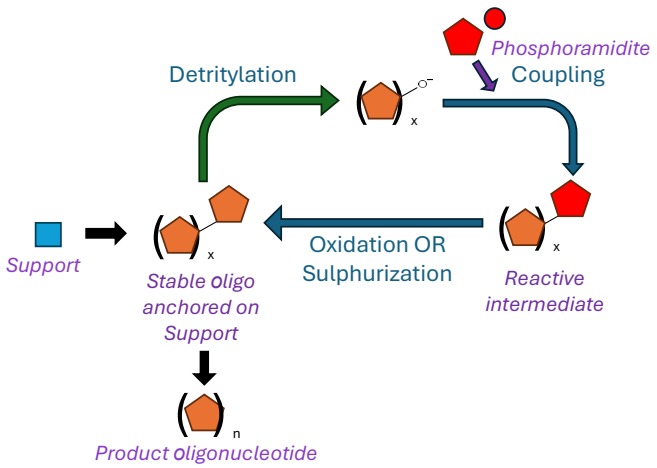


Figure 3: The synthesis cycle for oligonucleotides: Steps carried out in acetonitrile shown with blue arrows, in toluene with green arrows. The red circle represents the DMT protecting group.



Oligonucleotides act directly on the genome to upregulate or downregulate the production of a protein that is responsible for the symptoms of a disease [3, 4]. As of August 2024, there are currently 21 approved nucleic acid-based therapies [2], with over 50% of these approved within the last 5 years. Although most approvals have been for relatively small patient populations, there is a trend toward higher prevalence indications such as LEQVIO (inclisiran), which was approved in 2020 for cholesterol reduction[3]. At the same time, the rare—and specifically nano rare—disease applications are also growing, especially in the personalized medicine following the example of milasen, which was approved for personalized use in 2018 [5].

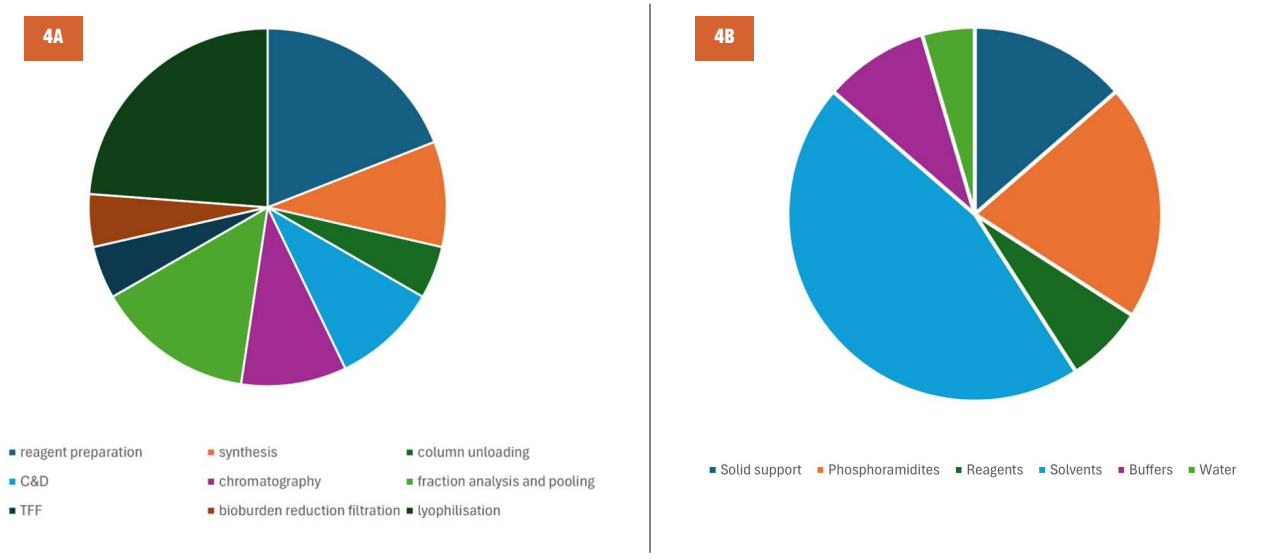
THE OLIGONUCLEOTIDE MANUFACTURING PROCESS

The manufacturing process for oligonucleotide drug substance is described at a high level in Figure 2. Depending on the mode of action of the oligonucleotide, the drug substance can be an antisense oligonucleotide (ASO), which is a single strand of nucleotides, or single interfering RNA (siRNA), which is a double strand linked by Watson-Crick pairing. Both ASO and siRNAs can be conjugated, for example, using GalNAc ligands, which are used to target delivery to the liver [3].

The synthesis steps are common to both ASOs and SiRNAs and consist of reagent preparation, synthesis, then cleavage and deprotection. These steps are run in acetonitrile and toluene, so the facility design must be suitable for use of large volumes of solvent.

The synthesis method for oligonucleotides was developed in the 1980s by the Caruthers group [6] and involves sequential addition of nucleoside phosphoramidites through an iterative synthesis cycle, as shown in Figure 3. The order of addition of the different phosphoramidites provides the base sequence and required ribose sugar modifications on the nucleotide chain. The synthesis cycle is conducted on a solid support packed in a column to enable excess reagents to be flushed away in a column rinse at the end of each

Figure 4: A: Approximate process timeline for the different manufacturing steps involved in oligonucleotide manufacture. B: Cost breakdown of the raw materials required for oligonucleotide manufacture assuming DNA with in-house buffer manufacture.



synthesis step. This approach has been evolving over the past few decades to improve yields and purity and to remove toxic solvents such as dichloromethane [7].

As only one nucleotide base is added per synthesis cycle, the phosphoramidites are protected with a dimethoxytrityl (DMT) protecting group. This prevents multiple nucleotide additions in each synthesis cycle. The protecting group is removed in the detritylation step readying the growing oligonucleotide chain to react with the next phosphoramidite addition. The detritylation step requires a change in solvent from acetonitrile, used in the coupling and oxidation steps, to a toluene-dichloroacetic acid solution, as shown in Figure 2. These washing steps to change over between solvents contributes significantly to the solvent consumption of the process.

The process development for the automated synthesis reactions can be complicated, with multiple parameters to be optimized for each of the four chemical steps in each of the 20 nucleotide couplings. Although there is platform knowledge to draw from, there is still sequence-specific optimization required in both the synthesis and purification methodologies. In addition, the quality of the reagents must be carefully controlled to provide optimum synthesis conditions. Water must be excluded from the synthesis reagents and reaction to avoid loss in yield, requiring use of ultra-dry solvents and control of moisture ingress, for example, by maintaining low humidity environments for manual operations.

The grown oligonucleotide is then removed from the solid support, and the remaining protecting groups are removed by a cleavage and deprotection reaction typically carried out in ammonium hydroxide to yield the crude oligonucleotide in solution. At this step, the crude oligonucleotide contains a number of impurities from failed coupling steps resulting in "shorter" impurities, synthesis errors, and impurities from side reactions. These numerous and closely related impurities can only be separated by chromatography.

There are several different strategies for purification, including use of ion exchange chromatography or reverse-phase purification relying on difference in charge and hydrophobicity, respectively. Whichever methods is used, separation of closely related impurities, for example an 18-mer from a 19-mer oligonucleotide, is challenging, and a trade-off between yield and purity is required.

A desalting or buffer exchange step is then required to produce the oligonucleotide solution in water. The solution is then lyophilized to give the oligonucleotide drug substance. The drug substance is then redissolved in the drug product process, excipients are added, and it is fill finished into vials or prefilled syringes. The typical administration route is via intravenous injection and, for some central nervous system indications, by intrathecal injection. Therefore, careful control of cross contamination via design of the manufacturing facility, process and choice of components are required to ensure the product is safe and efficacious.

MANUFACTURING PROCESS HOTSPOTS AND IMPROVEMENTS

Timeline and Cost

The approximate time and cost breakdown required to complete a batch of oligonucleotide drug substance is shown in Figure 4. This is for a sequence of DNA, and the cost contribution of the phosphoramidites will increase significantly for the more highly modified phosphoramidites. The cost contribution also assumes that buffers are prepared on-site from water generated in the facility. This step would occur in parallel with other steps in the manufacturing timeline, so although it does not appear on the timeline, it does consume significant operator resources and

manufacturing footprint, with some analysis showing that it can account for 20% of the overall cost of manufacture [8].

The cost breakdown in Figure 4B also does not include the cost of the chromatography resin or single-use consumables. For campaign manufacture, repeated use of the chromatography resin can be considered, but for clinical-phase manufacturing of just one or two batches of different oligonucleotide in each campaign, the repeated purchase cost of the chromatography resin will be another significant contribution.

Process Mass Intensity

From the example cost comparison, solvent is a key cost contributor and is also the major contributor to the process mass intensity. A typical oligonucleotide process requires 1,500 kg/solvent per 1 kg product [9]. For a maximum scale of 20 kg, 30,000 kg of solvent is required per batch. With some indications for oligonucleotide therapeutics demand expected to exceed 1 ton per year, it can result in significant strain on the solvent supply chain, particularly for acetonitrile.

The quantity and quality required for the acetonitrile is a significant demand compared to the market capacity. For example, for just one product at 1 ton per year demand, approximately 1,000 tons per year of ultra-dry acetonitrile are required, which is approximately 0.5% of the global acetonitrile market. Therefore, solvent recycling is an attractive option to reduce both the process mass intensity and exposure to a constrained supply chain. The waste acetonitrile–toluene solvent mixture is challenging to separate due to a number of ternary azeotropes, requiring complex recovery methods [10].

Process Steps

As shown in Figure 2, the oligonucleotide process is a mixture of recipe-driven, highly automated steps interspersed with manual processing steps. This requires careful process scheduling to allow for aging of some reagents while using other reagents within their optimum stability windows. Integrating typical plant control systems with the automated synthesizer is required to ensure a continuous supply of solvents and reagents, as well as adequate waste vessel capacity to avoid interruption of the synthesis process.

Fraction Analysis

Following the cleavage and deprotection reaction, the chromatography step results in several fractions, each requiring complex analytical processing to decide which fractions meet the required purity and specifications while still maintaining yield. In a manufacturing environment, the complexity of analyzing for the multiple closely related impurities is challenging and time consuming. While the analysis is being carried out and decisions made, cold storage is typically required for the fractions. This adds another adjacency requirement to the facility design to enable movement between controlled processing area and cold storage.

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Preparation

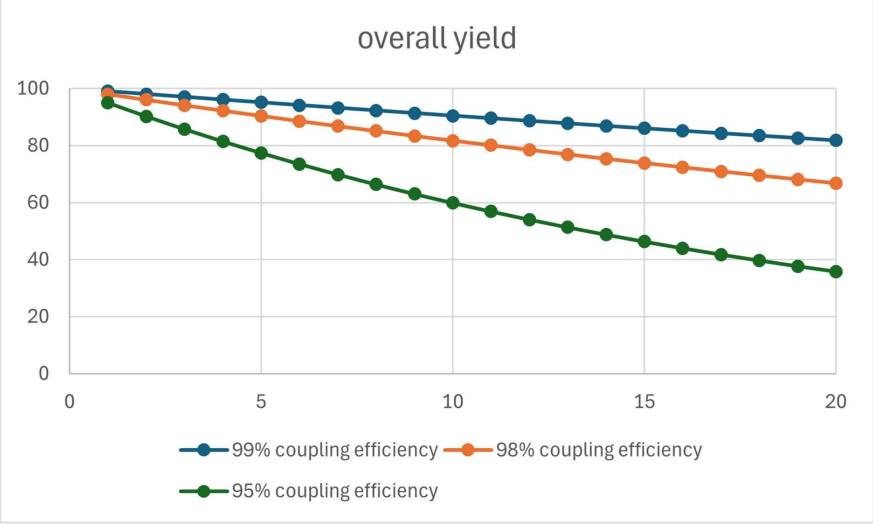
Various options for off-site or automated column preparation and buffer preparation have been developed for the biologics supply chain, and these manufacturing improvements can also be implemented for oligonucleotides.

When including the buffer preparation, column packing, and analytical steps, the downstream purification is a significant portion of the manufacturing time and footprint. Two or more chromatography steps can be required, depending on the manufacturing strategy chosen for addition of conjugating ligands and whether the molecule is an ASO or siRNA. Therefore, efforts to increase chromatography productivity already developed in other biopharmaceutical applications are being applied to oligonucleotides.

Intensified approaches to chromatography include continuous multi-column technology where case studies show reductions in cost and improvements for productivity [11]. There is also scope for on-line fraction analysis and for use of modeling to better understand and optimize the chromatography step. Modeling approaches will also allow for more rapid development of the chromatography step.

The oligonucleotide drug substance is used in parenteral products. According to the EMA guidance [12] the water used in the final purification steps must be purified water with appropriate specification for endotoxin and microbiological quality of the drug substance. Recently there has been a move in the industry to consider the oligonucleotide drug substance in a solution. This is because the lyophilization step is a time- and energy-intensive process. Removal of the lyophilization control point increases the level of control required on the water, and it would be expected that water for injection be used [12]. The oligonucleotide drug substance solution will typically be stored and shipped frozen to its next drug product processing steps [13, 14].

Figure 5: Theoretical yield across the iterative synthesis with coupling efficiencies of 99%, 98%, and 95%.



Production Scale and Batch Size

The column-based solid-phase synthesis approach allows for rapid preparation of oligonucleotides at smaller scales for screening and early preclinical testing. It is also a robust manufacturing method for the smaller-volume rare disease indications. There is a maximum scale determined by the physical limitations of maintaining consistent flow through the column. This maximum scale limit is in the region of 20 kg, depending on the loading achieved by the solid support, length of the oligonucleotide, and degree of optimization of the synthesis process. There is also a maximum batch size determined by the raw material value going into the batch, which with highly modified phosphoramidites could be into the multimillions of dollars. For larger-volume indications, the current solution is to scale out. This shifts the manufacturing challenge to one of planning, executing, analyzing, and releasing up to 100 batches a year.

FUTURE DIRECTIONS

Removal of the solid-supported, column-based approach would remove the scale limitation in the synthesis step and enable manufacturing equipment more like typical small molecule products to be used. This would then replace the scale limitation with a batch value limitation. Keeping the phosphoramidite-based cyclical chemistry requires the issue with clearing excess reagents between synthesis cycles to be solved by alternative means. Approaches to achieve this "clean-up" step in a liquid-phase system have been developed: for example, precipitation of the growing oligonucleotide chain between addition cycles and phase transfer between cycles [15, 16]. A single liquid-phase approach—using solvent-stable nanofiltration membranes to allow excess reagents to be washed from the reaction medium—has been identified and is currently being developed through a collaborative effort involving the UK government and industry support [17]. These approaches are suitable for peptide as well as oligonucleotide synthesis, supporting the extent of development ongoing in this area.

The liquid-phase approach can remove the scale limitation from the synthesis step and can increase opportunity for more facile solvent recovery and recycling. But with the fundamental iterative synthesis approach, there is a ceiling on the yield and purity. The downstream purification following these methods is likely to look very similar, as separation of the related impurities will still be required, so scale up of the chromatographic purification could become the new bottleneck in the process.

As shown in Figure 5, with a 99% coupling efficiency, the iterative synthesis will give at best a yield of 82% after 20 cycles, and this drops significantly with reduced coupling efficiency. As an alternative to the iterative linear approach, a more convergent fragment approach is being investigated. Fragments of 5–8 nucleotides chain length are synthesized, giving a yield more than 92% per fragment. There are different ligation approaches being developed to join these fragments together, including using the complementarity between strands. This could avoid the need to purify each strand [19], which would increase manufacturing output of the facility.

A step change in the production of oligonucleotides would be to move completely away from the phosphoramidite chemistry route and instead use enzymatic synthesis to build the oligonucleotide chain. Enzymes are being engineered to enable this synthesis, with the challenge being to find an enzyme (or panel of enzymes) that is suitable to cope with the diversity of modifications required for the therapeutic effectiveness of oligonucleotides. The advantage of an enzymatically catalyzed method is the replacement of solvent

by an aqueous environment, and a reduced need for protecting groups dramatically improves the process mass intensity of the process [20].

The enzymatic methods either involve the sequential addition of single nucleotides to a growing chain [21] or a templated one-pot process [20]. The sequential method uses a similar chain-growing cycle to the phosphoramidite chemistry and requires a "clean-up" step between cycles and a protected monomer to avoid multiple chain extensions in one cycle, whereas the one-pot approach uses base complementarity to control the base sequence. However, the base complementarity cannot differentiate between the sugar modifications on the nucleotide, so it is expected that a fragment approach would be required to account for the range in sugar and backbone modifications present on most therapeutic oligonucleotides. With both approaches improving atom efficiency with fewer protecting groups, enabling aqueous synthesis and conducting synthesis with high fidelity, there is great promise of a more scalable manufacturing method that potentially eliminates the requirement for chromatographic purification.

CHALLENGES

To harness the improvements possible from new technologies, there will be some challenges in switching over from the current solid-supported synthesis method.

Supply Chain

The current supply chain for phosphoramidites has responded to demand, but tight control of the impurities in the phosphoramidites starting materials is still required. For enzymatic synthesis, a new supply chain at the correct scale with the same control on purity will be required for the nucleoside precursors.

Manufacturing Base

With new technologies diversifying away from the platform solid-supported synthesis, the internal and external manufacturing supply base will need to provide a path through to commercial-scale manufacturing for both fragments and full-length oligonucleotides using solid-supported, liquid-phase, and enzymatic synthesis. There may be a specialization by different contract manufacturing organizations with these different technologies, which may require the pharmaceutical companies to review their manufacturing strategy.

Impurities in the Drug Substance

Particularly with enzymatic synthesis, there is likely to be a different impurity profile. So, the point in clinical development at which the manufacturing technology is changed over must be carefully evaluated against the comparability of the product yielded by the different methods. One challenge is the diastereomeric purity of

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certain oligonucleotides, which can be markedly different between chemically and enzymatically synthesized methods.

Molecular Complexity

As the sugar modifications and backbone chemistry of oligonucleotides increase in complexity, it could be more challenging for enzymes to accept these nucleoside precursors so further rounds of evolution would be required.

De-Risking of New Technologies

Demonstration at scale and in a pharmaceutically relevant environment will be required to give confidence to pharmaceutical companies to change their manufacturing strategy and for contract development and manufacturing organizations to invest in new technologies when there is already a growing customer base for solid-supported synthesis of oligonucleotides.

CONCLUSION

There will be challenges in implementation of the new technologies into the clinical and commercial supply chain for oligonucleotides. But given the opportunity and need for these new technologies, we will expect to see these changes being implemented as the new molecules make their way through the clinical pipeline. The challenge for the manufacturing base will be to make these medicines for undruggable targets at costs and resource impacts acceptable to the healthcare payers.

The manufacturing base for oligonucleotides has already significantly improved the process such that multi-kilogram batches are common and large-volume products become possible. The continued challenge will be to keep the improvement going across both synthesis and purification; the two parts of the process need to keep improving together.

Oligonucleotides are a challenging modality to manufacture, but by using the improvements in both the chemical synthesis and downstream purification, we are already seeing these therapies reach more patients, and this trend is set to continue. Interestingly, the challenge of manufacture exists both for increasing large-scale production and for small-scale, quick-turnaround personalized medicines. To meet these very different requirements, a new toolbox of manufacturing technologies must be developed.

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